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Isolation and 16S DNA Characterization of Soil Microorganisms from Tropical Soils Capable of Utilizing the Herbicides Hexazinone and Tebuthiuron

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ABSTRACT

Six non-fermentative bacteria were isolated from Colombian (South America) and Hawaiian (USA) soils after enrichment with minimal medium supplemented with two herbicides, hexazinone (Hex) and tebuthiuron (Teb). Microscopic examination and physiological tests were followed by partial 16S DNA sequence analysis, using the first 527 bp of the 16S rRNA gene for bacterial identification. The isolated microorganisms (and in brackets, the herbicide that each degraded) were identified as: from Colombia, *Methylobacterium organophilum* [Teb], *Paenibacillus pabuli* [Teb], and *Microbacterium foliorum* [Hex]; and from Hawaii, *Methylobacterium radiotolerans* [Teb], *Paenibacillus illinoisensis* [Hex], and *Rhodococcus equi* [Hex]. The findings further explain how these herbicides, which have potential for illicit coca (*Erythroxylum* sp.) control, dissipate following their application to tropical soils.

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Key Words: Hexazinone; Tebuthiuron; Pesticide degradation; Microbial isolation; Microbial identification; *Methylobacterium*; *Microbacterium*; *Paenibacillus*; Tropical soils.

INTRODUCTION

The annual economic costs of illicit drug abuse for the United States, in the year 2000, were ~ $50.2 billion in direct costs and ~ $110.5 billion in indirect costs.[1] For that same year, the retail value in the U.S. of the three major illicit drugs—cocaine, heroin, and marijuana—was estimated as $35.3, $10.0, and $10.5 billion, respectively.[2] Source supply reduction, a goal of U.S. drug control strategy, is targeted in part at eradicating three main drug crops: 1) coca (*Erythroxylum* spp.), source of cocaine; 2) opium poppy (*Papaver somniferum*), source of heroin; and 3) cannabis (*Cannabis sativa*), or marijuana. The first two are grown exclusively outside the U.S. Large plantings, or those in remote areas, are most efficiently controlled by aerial application of herbicides. In the largest such program, in Colombia (primary source of cocaine and heroin entering the U.S.), 122,695 ha of coca and 3,043 ha of poppy were sprayed with glyphosate in 2002.[3]

Whether used on weeds in, e.g., conventional agriculture or forestry, or against an illegal crop such as coca, the herbicides have undergone environmental and toxicological evaluations that contribute to overall risk assessment. One such parameter is persistence of the applied pesticide in soil, since this can influence the potential for surface or ground water contamination, and also the period of efficacy. Rarely, a pesticide adversely impacts nontarget soil microflora, affecting beneficial processes such as nitrification. Most often, however, the main influence is the microbial community's effect on the pesticide, i.e., through direct or co-metabolism of the pesticide by one or more members of that ecosystem. This is the basis for soil metabolism studies (usually with ^{14}C-labeled pesticide), conducted as a routine part of the pesticide registration process in the U.S. and elsewhere. Isolation and identification of microorganisms actually associated with breakdown is difficult and seldom done, yet provides proof that the pesticide of interest can be metabolized within specific soils. Most such studies have been done with temperate zone soils. The research described herein focuses on just such isolation and identification, for two herbicides with special interest to the control of narcotic plants.

Hexazinone is used for weed control in alfalfa, blueberries, pineapple, sugarcane, oil palm, rubber, tea, coniferous plantings, rights-of-way and other non-cropped areas.[4,5] Tebuthiuron is used for brush control in pasture, rangeland, rights-of-way and industrial sites.[5,6] These herbicides were introduced commercially in the mid-1970s. As with glyphosate,[7] ARS-USDA testing has found that hexazinone (Hex) and tebuthiuron (Teb) have high potential for coca control.[a] In addition to greenhouse experiments, these tests of Hex and Teb were conducted on illicit coca growing in Peru

[a]Most ARS-USDA research on herbicides to control drug crops has been published only as internal technical reports. Studies on coca control by hexazinone and tebuthiuron were mainly done in ca. 1987–1995 and on poppy control by hexazinone, in ca. 1997–2000.

and Panama, and at a controlled field site in Hawaii. Environmental sampling to monitor persistence and leaching in soil gave consistent results, with apparent half-lives of ca. 0.8–1 month for hexazinone and ca. 3–5 months for tebuthiuron.[8]a Dissipation was considered to be by microbial degradation, since leaching was limited. Earlier research had also demonstrated biological degradation for hexazinone[5,9–11] and tebuthiuron.[5,12] Furthermore, their disappearance was significantly faster than was typical for temperate regions,[13] indicating that environmental behavior in these tropical soils differed from the trends generally reported.

Recently, we briefly described related environmental research on Hex and Teb, showing that bacteria isolated from tropical soils of Hawaii and Colombia could survive on either Hex or Teb as sole carbon and nitrogen sources, and that loss of Hex was much faster than of Teb.[14] The present paper identifies in detail the methods and findings concerning the specific identification of those Hex- and Teb-degraders.

16S rRNA sequence information has been used for phylogenetic placement and identification of different physiological groups of bacteria, including such methylotrophic proteobacteria as *Methylobacterium* species.[15–18] Since our goal was to determine—if possible—the bacteria, in tropical soils, that were capable of surviving and utilizing either hexazinone or tebuthiuron as a carbon source, the investigational approach involved 1) isolating soil microorganisms that could metabolize Hex or Teb; and 2) identifying those predominant organisms by genetic analysis, using PCR sequencing of 16S ribosomal DNA (rDNA). A new genotypic identification system, the MicroSeq 500 16S bacterial sequencing kit (Perkin–Elmer Biosystems; Foster City, CA, USA),[b] is designed to sequence the first 527 base pairs (bp) of the 16S rRNA gene for bacterial identification;[19] Use of this system, with two sequencing primers to analyze a single PCR product, is described herein.

MATERIALS AND METHODS

Soils

Soils used for microbial enrichment were Halii sandy clay loam (Typic Gibbshumox) from Kauai, Hawaii, USA [22° 5′ N; 159° 24′ W], and an unclassified clay loam soil from Piamonte Municipality, Cauca Department, southwestern Colombia [1° 2′ N; 76° 9′ W]. Both soils were reddish-brown Oxisols. The Hawaiian soil was collected near a coca field test site. The Colombian soil was from an illicit coca field, recently eradicated and now under vigorous natural regrowth. Both were surface soils (0–20 cm), stored at ambient temperature and at the original field moisture content.

Soil Enrichment and Bacterial Isolation

A Basic Mineral Salt Medium (BSM) [K_2HPO_4, 3.5; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.27 (pH 7.3); $Fe_2(SO_4)_3 \cdot 9H_2O$, 0.03; and $CaCl_2 \cdot 2H_2O$, 0.03 g L^{-1}] was prepared

[b]Commercial products mentioned herein are solely for the readers' convenience and do not constitute endorsement by the U.S. Department of Agriculture over comparable products or sources.

and sterilized. Iron sulfate was filter-sterilized and added to the medium after autoclaving to prevent the formation of precipitates. To enrich for microorganisms capable of utilizing hexazinone and tebuthiuron, as a source of carbon and energy, the BSM was amended with either Hex or Teb (500 mg L^{-1} in water) to form a hexazinone- or tebuthiuron-selective medium (HSM or TSM). Technical-grade (100 ± 0.5% pure) hexazinone and tebuthiuron (AccuStandard; New Haven, CT, USA) were used throughout the experiments.

Microbial enrichments were initiated by the addition of 2 g (air-dry equiv.) of each soil into three 250-mL Erlenmeyer flasks containing 40 mL of either HSM or TSM. The flasks (with cotton plugs) were incubated aerobically in a G-24 environmental incubator (New Brunswick Scientific Co.; Edison, NJ, USA) at 26°C; aeration was ensured by constant shaking (125 rpm). After 9 days, 5 mL of each of the soil-medium enrichments was inoculated onto 20-mL Columbia bacterial agar (Difco Laboratories; Detroit, MI, USA) plates, supplemented with 500 mg L^{-1} of either HSM or TSM. This process was repeated twice. After the fourth enrichment, cultures of pure colonies were picked and transferred in replicates onto either 1000 mg L^{-1} HSM- or TSM-amended agar plates. Following incubation aerobically at 26°C for 7 days, isolated colonies were selected and sub-cultured onto agar plates of 500 mg L^{-1} HSM or TSM as well as onto Difco nutrient agar to ensure purity. Isolates were maintained by monthly transfers onto slants that contained HSM and TSM, and after growth, stored at 4°C. When desired, cells from the slants were inoculated into 10 mL of Difco nutrient broth in a 50-mL Erlenmeyer flask, grown aerobically, and subsequently utilized for the characterization studies.

Characterization of Isolates

Standard procedures were used to establish the cellular and colonial morphology of each isolate. After Gram-stain testing of the pure isolates, they were imaged using an Optronics (Goleta, CA, USA) digital camera. Selection of the organisms to be identified was based mainly on the three predominant colonies from each (Colombian or Hawaiian) soil enrichment.

Genotypic Identification

The MicroSeq 500 16S bacterial sequencing kit contains a PCR and cycle sequencing module, bacterial identification and analysis software, and a 16S ribosomal DNA sequence database library. Bacterial genomic DNA isolation and PCR amplification of the first 527 bp of the 16S rRNA gene[19] were performed according to the manufacturer's instructions with eubacterial universal primers 27F and 1492R.[20] Double-stranded sequence analysis of the first 527 bp was performed using an ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an Applied Biosystems ABI PRISM 377 or 310 DNA sequencer (Perkin–Elmer). Polymorphic positions present in those organisms containing multiple copies of the gene were included to ensure the highest degree of accuracy.[21]

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Genomic Fingerprints

Rep-PCR fingerprinting patterns from bacterial genomic DNA were generated with BOX1 primer.[22] Rep-PCR was performed in 50 μL (total volume) reaction mixture containing 2 μL lysed cells, 30 pmol of primer BOX1, 50 mM KCl, 20 mM Tris-HCl (pH 9.0), 3.0 mM $MgCl_2$, deoxynucleoside triphosphates (Amersham Pharmacia Biotech; Piscataway, NJ, USA) each at a concentration of 200 μM, and 1 U of Taq polymerase (Promega Corp.; Madison, WI, USA). The reactions were carried out in a Perkin–Elmer 9700 DNA thermal cycler: 1 cycle at 95°C for 6 min followed by 30 cycles at 94°C for 1 min, 53°C for 1 min, 65°C for 8 min, 68°C for 2 min, and a final extension at 65°C for 15 min prior to cold storage at 4°C. Rep-PCR product samples were separated by electrophoresis on horizontal 1.5% (w/v) agarose gels. Using the MicroSeq microbial identification and analysis software, as well as the BLAST program[c] to determine the closest available database sequences, sample files were assembled, and the final consensus sequence was compared with over 1,100 validated 16S ribosomal DNA sequences in the MicroSeq database library. Published sequences were obtained from GenBank.[d] Selected rDNA sequences were aligned and a phylogenetic tree was constructed using the Clustal W program by distance matrix analysis and the neighbor-joining method.[23,24] Bootstrap analysis was used to provide statistical confidence for the tree branch points. Phylogenetic trees were displayed using TREEVIEW.[25] Compared to the conventional phenotypic method, MicroSeq provided identical genus-level identification for all isolates.

RESULTS AND DISCUSSION

The methods used for microbial enrichment and isolation [e.g., the selective media and the high concentrations (500 and 1000 mg L^{-1}) of herbicide] precluded contamination of the final isolates by organisms that may have survived initial enrichments by catabolism of the scores of bacteria that initially would have been present. Ultimately, only those few that could utilize Hex or Teb as their carbon and nitrogen source survived. Although neither herbicide is toxic to soil microflora, at normal use rates,[26,27] Teb at these very high enrichment/isolation rates may have inhibited soil nitrifiers.[27]

Identification of the six principal bacterial isolates through genetic fingerprinting techniques is summarized in Figures 1–6. These phylogenetic trees, or dendograms of relatedness, are based on comparisons of the unknown isolate with known bacterial

[c]BLAST® (Basic Local Alignment Search Tool) is a set of search programs for exploring protein or DNA sequence databases. It is operated through the National Center for Biotechnology Information (NCBI), at the National Library of Medicine, National Institutes of Health (NIH), Bethesda, MD, USA. Internet access can be made via the NCBI homepage (http://www.ncbi.nlm.nih.gov).
[d]GenBank is the NIH genetic sequence database collection of all publicly available DNA sequences.

strains. The standard of comparison is the sequence of 527 bp in rDNA. Percent genetic distance (% GD) in Figures 1–6 is the percentage of nucleotides that are different. For example, isolate C4238-#1 B con is most closely related to *Microbacterium foliorum* (2.76% GD). Each figure shows the 10 most-similar biological matches to the unknown isolate, which in Figure 1 represents a Colombian soil isolate that utilized hexazinone as its sole C and N source. With this method, similarity with a GD < 1.0% usually indicates a species match; thus, the organism was identified with high reliability at the genus (*Microbacterium*) level, but the species assignment (as *M. foliorum*) is less certain.

Comparative sequence analysis of the 16S ribosomal DNA of all selected isolates revealed the six most abundant colonizing strains to be: as hexazinone-utilizers– *Microbacterium foliorum* (Gram/+) [Colombia], *Paenibacillus illinoisensis* (Gram/+) [Hawaii] and *Rhodococcus equi* (Gram/+) [Hawaii]; and as tebuthiuron-degraders– *Methylobacterium radiotolerans* (Gram/−) [Hawaii], *Methylobacterium organophilum* (Gram/−) [Colombia], and *Paenibacillus pabuli* (Gram/+) [Colombia]. The ''common biological denominator,'' i.e., the bacterial genus isolated from both soils and capable of utilizing both herbicides equally, was *Paenibacillus*.

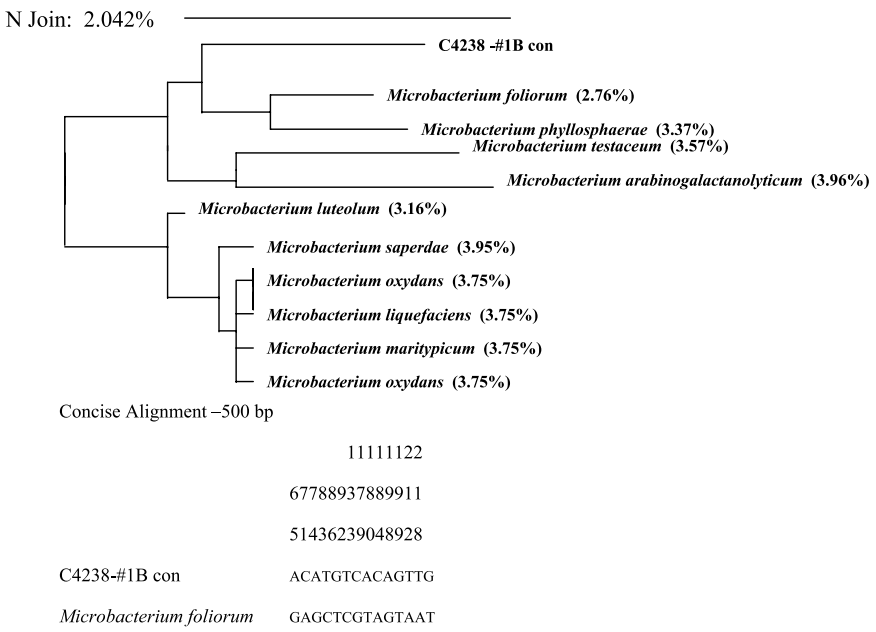

Figure 1. Phylogenetic tree showing the relationships among selected strains of *Microbacterium* sp. and published 16S rDNA sequences. The unknown bacterium (isolate C4238-#1B con) source: Colombian soil; herbicide degraded: hexazinone. The GenBank percent genetic distance (% GD) as well as the accession numbers are shown in parentheses. GD is the percent of nucleotides that are different; usually, < 1.0% indicates a species-level match. A similarity ranking (SR) > 0.95 usually indicates a species match.

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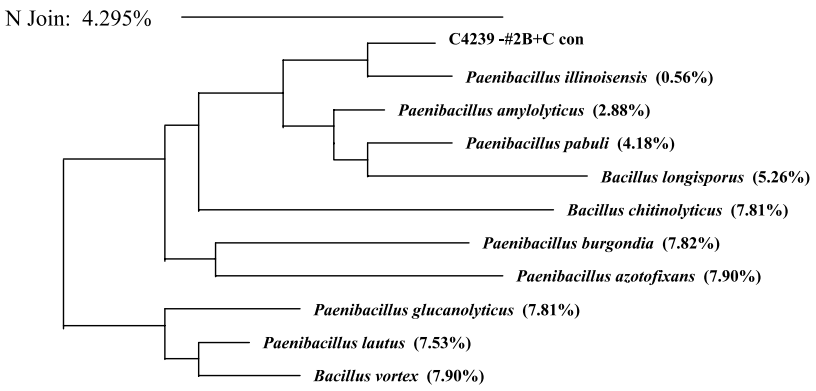
Except for *M. foliorum* and *P. pabuli* (GD = 1.58%; Figure 6), selected identifications were at GD 1.00% or smaller. The closest matches between unknown isolate and the assigned bacterial strain are illustrated on Figures 4 and 6. Sample C249-#36G-R con had a GD of only 0.21%; the Concise Alignment (lower part of Figure 4) shows that the unknown contained purine base A (adenine) at position 167, whereas *Methylobacterium radiotolerans* contained pyrimidine base T (thymine) here. Elsewhere, the nucleotide sequence was identical. Similarly, in Figure 6, isolate C251-#31G-R con was virtually homologous with *Methylobacterium organophilum* (0.21%), and differed only at nucleotide position 118 [C (cytosine) for the isolate, T for *Methylobacterium organophilum*]. The Hawaiian strain illustrated in Figure 3 was ranked at the same relatedness (1.00%) to both *Rhodococcus equi* and *Corynebacterium hoagii*. The 527-bp sequence was identical for *R. equi* and *C. hoagii*, so if those library standards actually differ, then it is within an unexamined section of their genomes.

Molecular heterogeneity among the tebuthiuron-degrading bacteria was investigated. As shown in Figure 4, and from rep-PCR fingerprinting profiles (not shown), the strains *Methylobacterium radiotolerans* and *Methylobacterium organophilum* are nearly, but not completely, homologous. The same observation was clear with *Paenibacillus pabuli* and *Paenibacillus illinoisensis* strains. The data also



Concise Alignment –500 bp

	2
	479
	025
C4239-#2B+C con	AAG
Paenibacillus illinoisensis	GTC

Figure 2. Phylogenetic tree showing the relationships among selected strains of *Paenibacillus* sp. and published 16S rDNA sequences. The unknown bacterium (isolate C4239-#2B + C con) source: Hawaiian soil; herbicide degraded: hexazinone.

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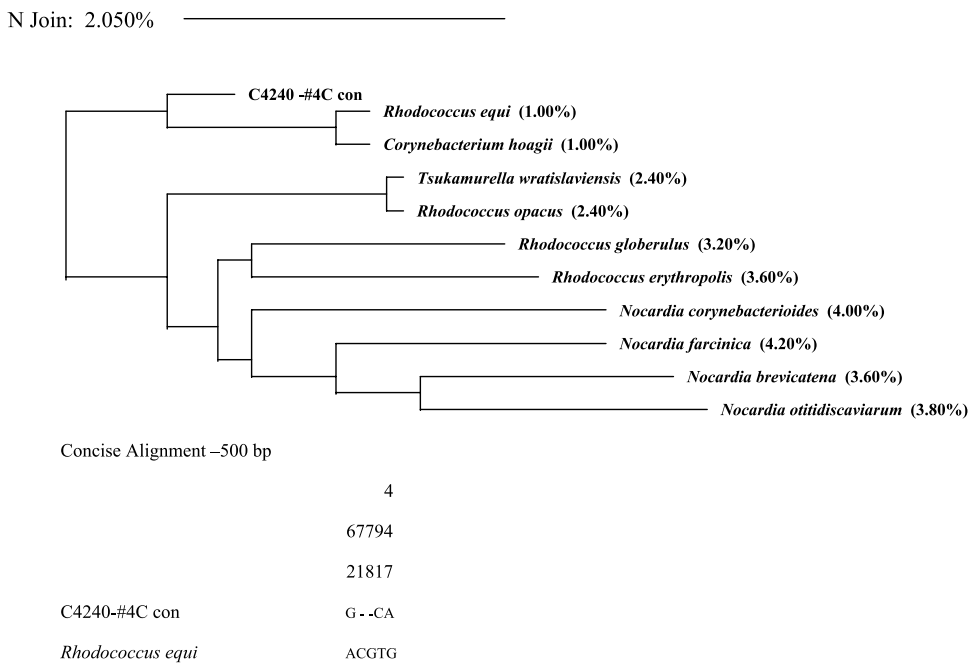

Figure 3. Phylogenetic tree showing the relationships among selected strains of *Rhodococcus* sp. and published 16S rDNA sequences. The unknown bacterium (isolate C4240-#4C con) source: Hawaiian soil; herbicide degraded: hexazinone.

show that on the genus level, both species were able to utilize Hex and Teb to varying degrees.

Methylobacterium

Methylobacterium spp. represented two of our six Hex-Teb isolates. This genus[28] is a group of strictly aerobic, facultative methylotrophic, Gram-negative, rod-shaped bacteria that are able to grow on one-carbon compounds more reduced than carbon dioxide as sole carbon and energy sources.[29] Mass cultures of these facultative methylotrophs are pink to red because of the presence of carotenoids. The genus *Methylobacterium* consists of nine species (Figures 3 and 6), with *M. organophilum* as the type species.[30,31] They are phenotypically and chemotaxonomically quite similar, and phenotypic differences among the species are found in only limited properties such as carbon source utilization.

Members of the genus *Methylobacterium* are distributed in a wide variety of natural habitats, including soil, dust, air, fresh water and aquatic sediments, and non-natural settings such as potable water supplies.[32] Of principal relevance to the present research, however, is the fact that *Methylobacterium* spp. have been isolated from soils.

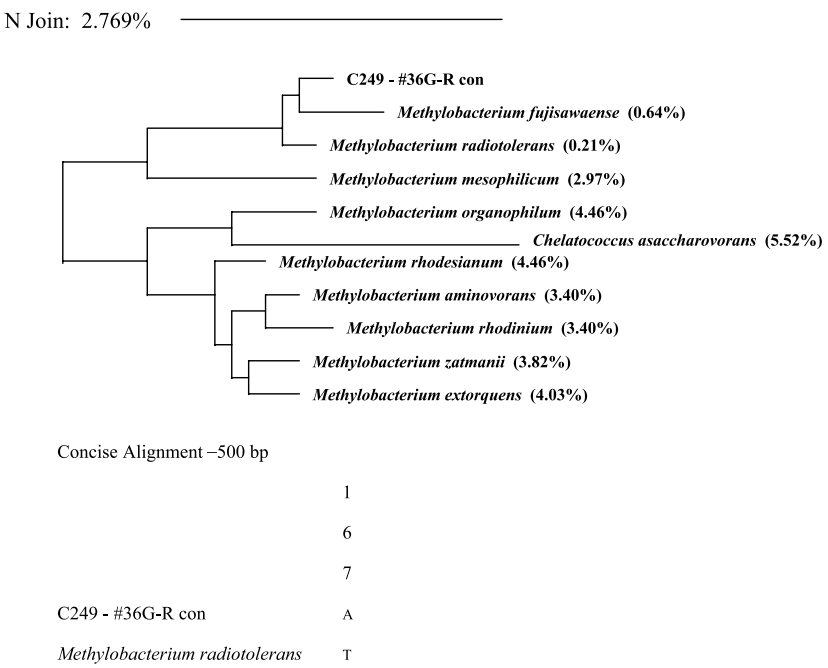

Figure 4. Phylogenetic tree showing the relationships among selected strains of *Methylobacterium* sp. and published 16S rDNA sequences. The unknown bacterium (isolate C249-#36G-R con) source: Hawaiian soil; herbicide degraded: tebuthiuron.

Both *Methylobacterium* species found in our research degraded tebuthiuron. Since *N*-demethylation is a major initial step in Teb metabolism in soils,[5,6] perhaps it is not coincidental that *Methylobacterium* is a genus specifically identified with the capability of metabolizing methyl moieties.[29]

Rhodococcus

Rhodococcus strains have been isolated from soil under conditions indicative of adaptation to xenobiotics, and in one interesting case, perhaps to narcotic plants. The latter was a strain of *Rhodococcus* isolated from the rhizosphere of *E. coca*; it was capable of utilizing cocaine as the sole source of C and N, for growth.[33] Strain *R. opacus* M213, from fuel oil-contaminated soil, could grow on simple one- and two-ring aromatic hydrocarbons.[34] Similarly, soil-isolated *Rhodococcus* sp. strain DK17 could utilize *o*-xylene as its sole C and energy source,[35] and also grew on other alkylbenzenes. Another *Rhodococcus* could metabolize methylated and methoxylated *s*-triazine ring compounds (from hydrolysis of sulfonylurea herbicides) as its only N source.[36] In our study, the isolated *Rhodococcus equi* was able to utilize the *s*-triazine herbicide hexazinone.

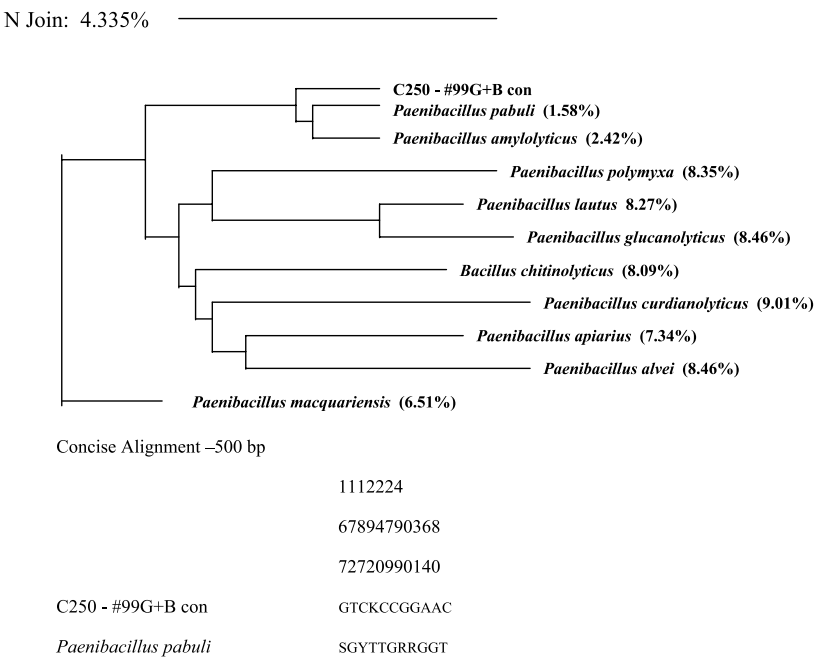

Figure 5. Phylogenetic tree showing the relationships among selected strains of *Paenibacillus* sp. and published 16S rDNA sequences. The unknown bacterium (isolate C250-#99G + B con) source: Colombian soil; herbicide degraded: tebuthiuron.

Microbacterium

Members of the coryneform (rod-shaped) genus *Microbacterium* are among the dominant thermoduric (heat-tolerant) microorganisms. For example, Gram-positive *Microbacterium* sp. were isolated from milk immediately after heating and following refrigerated storage.[37] Other *Microbacterium* spp. were obtained from activated sludge and soil samples that were capable of assimilating acrylic trimer as a carbon and energy source.[38] Perhaps the heat-tolerant characteristic gives an ecological advantage, enhancing the potential for *Microbacterium* spp. to be found in tropical soils. As is common for many pesticides, higher soil temperatures were associated with faster breakdown of both hexazinone[11] and tebuthiuron.[12]

Paenibacillus

Nitrogen-fixing *Paenibacillus* (formerly *Bacillus*) strains were present in latosolic (an Oxisol) and low-humic gley Brazilian agricultural soils.[39] In another study,[40] of 13 independent, nitrogen-fixing genotypes isolated from birch root surfaces and conifer forest humus in Finland, seven were identified (rep-PCR) as *Paenibacillus* sp., with similarities of 95.0–96.0% to *P. durus*. That taxon was in

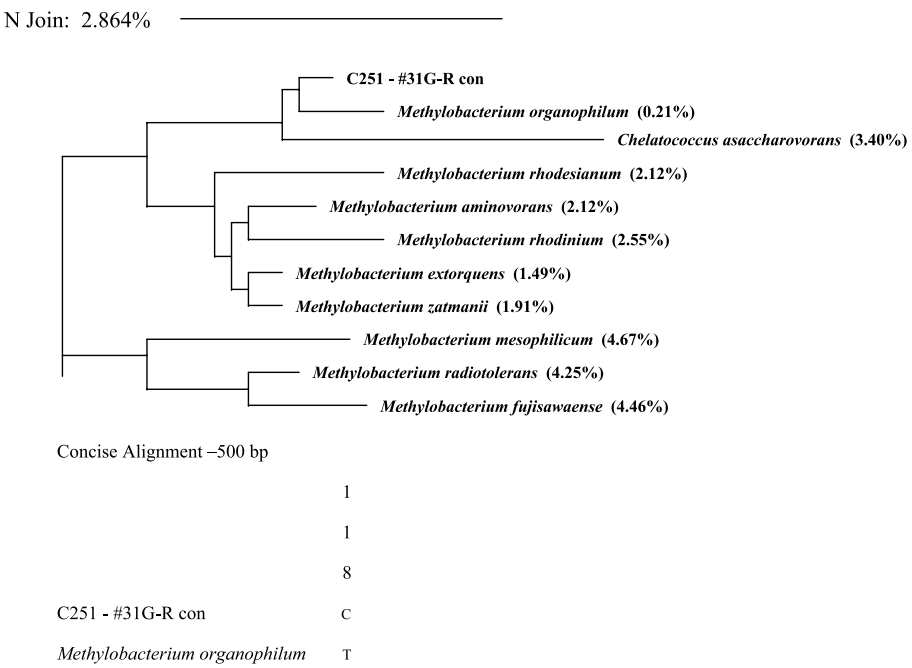

Figure 6. Phylogenetic tree showing the relationships among selected strains of *Methylobacterium* sp. and published 16S rDNA sequences. The unknown bacterium (isolate C251-#31G-R con) source: Colombian soil; herbicide degraded: tebuthiuron.

both rhizoplane and humus. Within the almost-full 16S r-DNA sequence, similarities among the seven *Paenibacillus* isolates were 97.3–100%, but < 98% similar to any sequence in the database used, indicating that they belonged to species not yet sequenced.

We isolated two *Paenibacillus* strains, *P. illinoisensis* (GD = 0.56%) and *P. pabuli* (GD = 1.58%), from Hawaiian and Colombian soil, respectively. The relative similarity between these strains and the nitrogen-fixing *P. azotofixans* (the type found in Brazil) can be seen in Figure 2. The Hawaii, Colombia, and Brazil (the latosol) soils share similarity in being highly oxidized and acidic.

Further Studies

Limited testing was done[14] with the purified soil isolates, grown to their lag-2 growth phase in liquid broth medium, then incubated aerobically (in light and in darkness) with 0 or 10 ppm Hex or Teb in BSM, BSM + N source, or BSM + C source. Hexazinone loss was unaffected by supplemental N, and only slightly retarded by added C; loss was rapid and unaffected by light. Additional investigations and analyses continue and may be reported later.

CONCLUSIONS

The potential coca-control herbicides hexazinone and tebuthiuron were shown, in earlier ARS-USDA research, to dissipate more rapidly from tropical field soils than was generally reported from studies done in temperate-zone soil. It was surmised that faster loss was biologically-mediated, a consequence of higher mean soil temperature and moisture content (the latter influenced by seasonal precipitation patterns). The present research confirmed the presence of bacteria, isolated from Colombian and Hawaiian soils, which could utilize the herbicides as sole carbon and nitrogen sources. Furthermore, the major organisms were positively identified, through rRNA analysis, as *Methylobacterium*, *Paenibacillus*, *Microbacterium*, and *Rhodococcus* spp. The first two genera, but with different species, were present in both sources of soil. We believe that our study represents the first identification of specific soil microorganisms that utilize/degrade hexazinone and tebuthiuron.

This research adds to the environmental assessment of two herbicides, but from the less-studied tropical perspective. Since the conditions of sample collection relate directly to those of most-likely use, it is clear that soil in the main regions of Colombia coca production contains microflora capable of metabolizing hexazinone or tebuthiuron. The Hawaii soil, which is basically similar, had (as expected) aerobic soil bacteria with the same capacity for Hex and Teb utilization; this helps explain earlier field losses of the herbicides applied to this research site.

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